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Serologic Responses by Immunoblot Following Natural Infection With Rotavirus Serotypes G1 and G4 in Children

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Serologic responses to proteins of rotavirus serotypes G1, P1A[8]; G2, P1B[4]; G3, P1A[8]; and G4, P2A[6] were evaluated by immunoblotting paired sera from 17 children with primary rotavirus infection. Ten children were infected with G1, P1A[8]; five with G4, P1A[8]; and two with G4, P2A[6] viruses. Anti-VP6 and anti-VP2 were seen in most responses. Homotypic anti-VP7 developed following G1 and G4 infections in 8 (80%) and 6 (86%) cases, respectively. Homotypic anti-VP4 developed in 9 (60%) cases following P1A[8] infection and in 0 of 2 cases following P2A[6] infection. Heterotypic anti-VP7 appeared against G4 (20%) and G3 (20%) following the 10 G1 infections, and against G3 (86%) and G1 (57%) following the 7 G4 infections. Heterotypic anti-VP4 occurred in only 3 (18%) children. The data show the antigenic predominance of internal proteins VP6 and VP2. Homotypic antibodies developed against VP7 but not against VP4 in most cases, while heterotypic antibodies were infrequent. ***J. Med. Virol. 56:52–57, 1998.***

KEY WORDS: pediatrics; viral infection; immune response

INTRODUCTION

Rotaviruses are the most common cause of dehydrating diarrhea in young children. A number of candidate vaccines designed to prevent severe rotavirus disease are currently being evaluated [Conner et al., 1994; Kapikian et al., 1996]. Development of vaccines has been hampered by the complexity of the immune response to rotavirus and the lack of known correlates of protection.

Rotaviruses have an 11-segment RNA genome that encodes for a number of structural (VP1 to VP4, VP6, and VP7) and nonstructural (NSP1 to NSP5) proteins arranged in a triple-shelled structure around a central core. The inner shell is formed by VP2; VP6 is the major intermediate shell protein carrying group and subgroup antigens, and VP4 and VP7 are the two main outer shell proteins. In addition, VP4 is posttranslationally cleaved to VP5* and VP8* [Bernstein and Ward, 1998].

Rotavirus serotypes are specified by VP4 and VP7 [Estes, 1996]. The presence of serotype-specific neutralizing antibodies to both proteins has been shown to be important in conferring protection against rotavirus infection [Hoshino et al., 1988]. Because the genes encoding these proteins segregate independently of each other during reassortment, a dual-serotyping system to account for the serotype specificities of both VP7 and VP4 has been adopted [Estes, 1996]. G-serotype refers to VP7, the outer capsid glycoprotein. Fourteen G-serotypes have been identified, but only four, G1–G4, are important causes of diarrhea in infants worldwide [Wyatt et al., 1983]. P-serotype designates VP4, the protein that activates rotavirus infectivity. Currently, 10 P-serotypes have been characterized, with serotypes 1A[8] and 1B[4] being the most common in infants with diarrhea [Gentsch et al., 1996].

Little information is available regarding the frequency with which antibodies develop against specific rotaviral proteins following natural infection. Svensson et al. [1987], using a radioimmunoprecipitation assay, found VP2 and VP6 most antigenic and anti-VP7 present in only 3 of 8 infected children. Richardson and Bishop [1990], using an immunoblot assay, assessed the homologous antibody response to G1 infection in 16 patients and found anti-VP2, -VP6, and -VP7 in all cases, and anti-VP4 in 67% of the cases. Using radio-

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immunoprecipitation, Richardson et al. [1993] studied 14 patients with G1 and 6 patients with G4 infection and described homologous and heterologous anti-VP2, -VP6, and -VP4 response in the majority of patients; the study found that homologous anti-VP7 was common following G1, but not G4, infection, and heterologous anti-VP7 was infrequent. In none of these studies was the P serotype of the infecting strain known.

The aim of the present study was to evaluate the contribution of the individual viral proteins in the serologic response, with special attention to the presence of anti-VP4 and anti-VP7 because of their potential for neutralization. We considered it important to evaluate the frequency with which antibodies are developed and the serotypes (homologous and heterologous) they are directed against, since that information will help decide the number of serotypes that need to be incorporated into an effective rotavirus vaccine.

MATERIALS AND METHODS

Patients, Stool Specimens, and Sera

Patients with rotavirus infection, as diagnosed by enzyme immunoassay (Premier Rotaclone, Meridian Diagnostics, Cincinnati, OH), had stools collected for serotyping. G-serotyping of rotavirus-positive stools was performed by an enzyme immunoassay employing VP7-serotype-specific monoclonal antibodies as previously described [Begue et al., 1992], and P-serotyping was performed by polymerase chain reaction (PCR) at the Centers for Disease Control and Prevention (Atlanta, GA) [Gentsch et al., 1992].

Each patient was assigned an illness severity score using the criteria outlined by Ruuska et al. [1991], which included the following: duration and maximum number of diarrheal stools, duration and maximum number of vomiting episodes, presence of fever and dehydration, and the need for treatment. The score ranges from 0 to 20, with 20 being most severe.

Informed consent was obtained from patient's parents or guardians. The study was approved by the Institutional Review Board of the Rhode Island Hospital (Providence, RI) and was conducted in compliance with guidelines of the U.S. Department of Health and Human Services.

After informed consent was obtained, sera was collected by venipuncture or heelstick during the acute (<5 days) and convalescent (2 to 10 weeks) phase of the disease and stored at −70°C or −20°C until tested. Sera was tested for neutralizing antibody at the James N. Gamble Institute of Medical Research (Cincinnati, OH) as previously described [Knowlton et al., 1991].

Immunoblotting

Rotaviruses of serotype G1, P1A[8] (strain WA); G2, P1B[4] (strain DS-1); G3, P1A[8] (strain P); or G4, P2A[6] (strain ST-3) specificity were propagated on MA104 cells, purified by extraction with trichlorotrifluoro-ethane (Freon), concentrated 200-fold by ultracentrifugation, and resuspended in Tris-buffered saline (0.02-M) with 10-mM $CaCl_2$ as described by Richardson and Bishop [1990]. Before use, each virus preparation was both G- and P-serotyped to verify its identity.

The virus preparation was then boiled for 5 min in the presence of 1% sodium dodecyl sulfate (SDS) and 50-mM dithiothreitol (DTT) (BioRad, Hercules, CA) to dissociate the proteins [Tsang et al., 1986] and 150 μl was subjected to electrophoresis in a 10% acrylamide gel [Laemmli, 1990]. The resolved proteins were then electrophoretically transferred onto 0.2-μm-pore-size nitrocellulose sheets (NCS) (Schleicher & Schuell, Inc., Keene, NH) by the procedure of Towbin et al. [1979] using a TE22 Mini Transphor system (Hoefer Scientific Instruments, San Francisco, CA). The presence of the viral proteins on the NCS was verified with hyperimmune rabbit antirotavirus sera specific for each serotype (provided by the Centers for Disease Control and Prevention).

Fifty μl of a 1:10 or 1:40 serum dilution was incubated with the NCS in a Miniblotter 28 (Immunetics, Cambridge, MA). The same dilution was used for each paired sera and was determined by the quantity of serum available. Briefly, 45–50 μl of serum diluted in milk buffer (phosphate-buffered-saline with 0.25% Tween 20 and 5% nonfat dry milk) was added to fill the channels of the miniblotter, where the NCS, wet in milk buffer, had been placed. After incubation for 1 hr at room temperature, the NCS was washed with milk buffer, removed from the miniblotter, and fixed with 9% formaldehyde. After washing, alkaline phosphatase-conjugated goat antihuman IgG (1:2000 dilution, Tago Inc., Burlingame, CA) or IgA (1:500 dilution, Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added, incubated for 15 min, washed, and the reaction was developed with 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP-NBT) (Kirkegaard & Perry Laboratories) for 10 min. Prestained molecular weight standards (GIBCO-BRL, Bethesda, MD) were used to identify each protein according to its migration site. The expected molecular weights (kDa) of the viral proteins were as follows [Kapikian and Chanock, 1985]: VP1 (125), VP2 (94), VP4 (84), VP5* (60), VP6 (41), VP7 (37), and VP8* (28). Uninfected cells underwent the same purification procedure and were blotted against patient serum showing a minimal background, not interfering with the proteins of interest.

Bands were scored visually as 1+ (barely seen), 2+ (well seen, faint), 3+ (well seen, strong), or 4+ (well seen, thick smear). A serologic response was considered to occur when new bands developed or a band increased in intensity in convalescent as compared with acute sera. Since some bands were rather faint and differences in intensity sometimes difficult to visualize, for more objective measures, each blot was also scanned using a pdi DeskTop Scanner (PDI, Inc., Huntington Station, NY) and the scans were analyzed on a Unix SPARC station computer using the pdi Quantity One, version 2.4, software package for analysis of one-dimensional gels. A positive result by scan was defined

TABLE I. Neutralizing Antibodies to Rotavirus Serotypes G1 Through G4 in Patients With Serotype G1 and G4 Infection

Age (months)	Illness score	Serum (day from onset)	Infecting serotype	Neutralizing titer against[a] WA (G1, P1A[8])	DS-1 (G2, P1B[4])	P (G3, P1A[8])	ST-3 (G4, P2A[6])	VA-70 (G4, P1A[8])
16	12	acute (1)	G1, P1A[8]	<5	11	<5	<5	<5
		convalescent (34)		N/D	N/D	N/D	N/D	N/D
20	8	acute (0)	G1, P1A[8]	<5	<5	<5	<5	<5
		convalescent (15)		483	34	40	<5	101
23	16	acute (1)	G1, P1A[8]	<5	<5	<5	<5	<5
		convalescent (33)		107	9	23	8	16
30	10	acute (1)	G1, P1A[8]	<5	11	<5	<5	<5
		convalescent (42)		1700	30	364	54	150
31	10	acute (1)	G1, P1A[8]	<5	7	<5	7	<5
		convalescent (32)		N/D	N/D	N/D	N/D	N/D
6	14	acute (-1)	G4, P1A[8]	<5	<5	<5	8	61
		convalescent (17)		61	16	108	36	181
16	10	acute (-10)	G4, P1A[8]	<5	<5	<5	<5	<5
		convalescent (7)		48	33	65	76	682
21	3	acute (-3)	G4, P1A[8]	<5	15	<5	<5	<5
		convalescent (8)		27	<5	39	12	77
56	13	acute (0)	G4, P2A[6]	<5	<5	<5	<5	<5
		convalescent (33)		20	26	19	268	177

[a]N/D = not done.

as a band of ⩾0.05 signal units. A serologic response by scan was considered to occur when new bands developed or a band increased in intensity by at least 0.05 signal units in convalescent as compared with acute sera. The 1+, 2+, 3+, and 4+ categories roughly corresponded to 0.1–0.4, 0.4–0.8, 0.8–1.6, and >1.6 signal units.

RESULTS

Seventeen patients with natural rotavirus infections were studied. Seven patients were infected with rotavirus serotype G1, P1A[8]; four with serotype G4, P1A[8]; and two with serotype G4, P2A[6]. Stool isolates from four patients were not available for P-serotyping. Since these isolates were electropherotipically identical to similar G-serotype isolates obtained from the same community during the same season, they were presumed to be of the same P-serotype (i.e., three G1, P1A[8] and one G4, P1A[8]). Patient ages ranged from 18 days to 56 months, with a median of 13.5 (range 2 to 31) months for serotype G1, P1A[8]-infected, 16 (range 6 to 22) months for G4, P1A[8]-infected, and 28.3 (0.5 and 56) months for G4, P2A[6]-infected subjects. The median interval between the two serum specimens was 33 (range 15 to 97) days for G1, P1A[8]-infected, 17 (range 12 to 22) days for G4, P1A[8]-infected, and 34.5 (33 and 36) days for G4, P2A[6]-infected infants, and the median illness severity score was 10 (range 8 to 16) for G1, P1A[8] infection; 12 (range 3 to 14) for G4, P1A[8] infection; and 11.5 (10 and 13) for G4, P2A[6] infection. Only one of the patients had an underlying disease (hypotonia).

Nine of the 17 patients had serum available for neutralization assays against serotypes G1–G4 (Table I). Except for one 6-month-old infant with an acute titer of 61 against VA-70, all other patients had neutralizing antibody titers of <20 against serotypes G1 through G4 in the acute sera. In the convalescent sera, all tested children seroconverted by neutralizing antibody response to the infecting serotype and developed low titers of neutralizing antibody response to the heterologous G-serotypes.

IgG and IgA Responses

Both IgG and IgA antirotavirus antibody responses were detected by immunoblot. Visual and scanned results showed a high degree of correlation. In general, IgG antibodies were directed against a greater number of proteins and were present in greater intensity than IgA antibodies. Most IgA responses had a corresponding, more intense, IgG response. The number and intensity of responses did not seem to correlate with the age of the patient, time of serum collection, or severity of the disease.

Serotype G1, P1A[8] Infections

Following rotavirus serotype G1, P1A[8] infection, serologic responses to the homologous virus (strain WA) occurred frequently and were directed against most viral structural proteins (Table II). IgG anti-VP6 and anti-VP2 antibodies were the most intense, present in the majority of patients. Homologous anti-VP4 and anti-VP7 antibodies were each detected in 8 (80%) of the 10 patients. Responses to heterotypic viruses were directed most often against VP6 and VP2. Also worthy of note is that heterotypic anti-VP7 antibodies developed against the G4 strain (ST-3) and the G3 strain (P) in two children for each strain. No child developed anti-VP7 antibodies against more than one heterologous serotype and only one child made heterotypic anti-VP4 antibodies. IgA antibodies appeared less frequently and were most often directed against VP6 of the homologous and heterologous strains. Homotypic anti-VP7 of the IgA class appeared in only 1 (10%) of the 10 cases, and anti-VP4 in 5 (50%) cases. A representative IgG response is shown in Figure 1. Each set

TABLE II. IgG and IgA Immunoblot Responses to Viral Proteins of G1–G4 Serotype Viruses Following Rotavirus Serotype G1, P1A[8] Infection in 10 Children

IgG (G-type/P-type) strain	Number responding to each protein[a]						
	VP1	VP2	VP4	VP5*	VP6	VP7	VP8*
WA (G1, P1A[8])	6	8	**7**	3	10	**8**	8
DS-1 (G2, P1B[8])		8	1	3	10		
P (G3, P1A[8])		10	**1**	1	10	2	
ST-3 (G4, P2A[6])		9		8	10	2	

[a]Numbers in boldface = homologous response.

IgA (G-type/P-type) strain	Number responding to each protein[a]						
	VP1	VP2	VP4	VP5*	VP6	VP7	VP8*
WA (G1, P1A[8])	2	3	**4**		7	**1**	4
DS-1 (G2, P1B[8])				1	3		
P (G3, P1A[8])		3	**1**		8		
ST-3 (G4, P2A[6])		2		5	7		

[a]Numbers in boldface = homologous response.

TABLE III. IgG and IgA Immunoblot Responses to Viral Proteins of G1–G4 Serotype Viruses Following Rotavirus Serotype G4, P1A[8] Infection in Five Children

IgG (G-type/P-type) strain	Number responding to each protein[a]						
	VP1	VP2	VP4	VP5*	VP6	VP7	VP8*
WA (G1, P1A[8])	3	5	**2**	1	5		
DS-1 (G2, P1B[8])	2	5	2	5	5		
P (G3, P1A[8])		5		4	5	5	
ST-3 (G4, P2A[6])		5		3	5	**5**	

[a]Numbers in boldface = homologous response.

IgA (G-type/P-type) strain	Number responding to each protein[a]						
	VP1	VP2	VP4	VP5*	VP6	VP7	VP8*
WA (G1, P1A[8])	3	4	**3**	3	5	3	
DS-1 (G2, P1B[8])	1	1	2		1		1
P (G3, P1A[8])		1		1	5		
ST-3 (G4, P2A[6])				1	3	**2**	

[a]Numbers in boldface = homologous response.

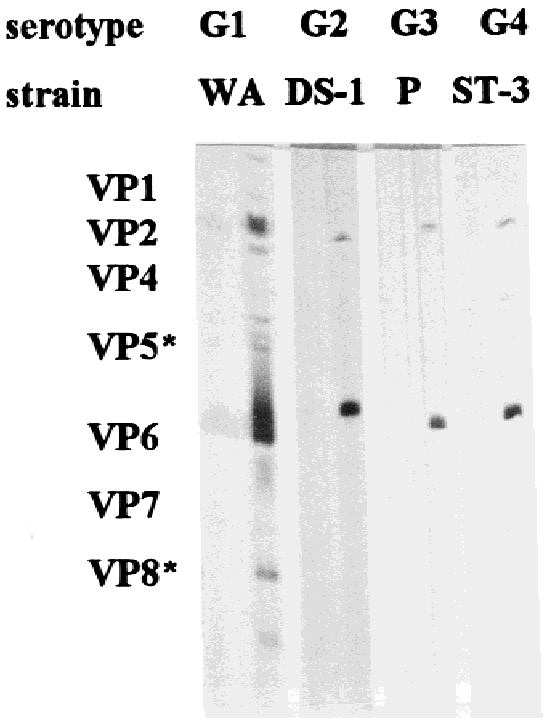

Fig. 1. Representative IgG responses following rotavirus serotype G1, P1A[8] infection. Note that anti-VP2 and anti-VP6 are present against all four serotypes. Anti-VP4 is present against serotype G1, P1A[8] (homotypic) and anti-VP7 is present against serotype G1, P1A[8] (homotypic) and G4, P2A[6] (heterotypic).

of two lanes represent acute and convalescent sera of the same patient, blotted against rotavirus strains representative of the four clinically significant G-serotypes.

Serotype G4, P1A[8] Infections

Following serotype G4, P1A[8] infection a different pattern of serologic responses was noted (Table III). For IgG, anti-VP6 and anti-VP2 responses were present against both the homologous and heterologous virus strains. Anti-VP7 developed in all cases against both the homologous G4 but also the heterologous G3 strain. Anti-VP4, on the other hand, developed against the homologous P1A[8] strains in only 2 of the 5 (40%) children, and against the heterologous P1B[4] strain in 2 children. IgA responses followed the same pattern as IgG, but the bands appeared in fewer number and less intensely. Figure 2 shows a representative IgG response.

Serotype G4, P2A[6] Infections

Following serotype G4, P2A[6] infection no clear pattern of serologic responses was noted (Table IV). For IgG, VP6 was the most antigenic protein, while VP2, VP5*, and VP7 responses were seen for both heterologous and homologous strains. An anti-VP4 response was seen against only one strain, G1, P1A[8] (WA), a heterologous virus. The oldest patient in the study, a 56-month-old child, had the broadest response of any patient responding to two of the three heterotypic G-viruses and 1 heterotypic P-virus. IgA responses were primarily directed at VP6.

DISCUSSION

The infecting G-serotypes studied (1 and 4) are the two most common in our region, comprising 82% of rotavirus infections [Begue et al., 1992]. Of the two P-serotypes studied, P1A[8] is the most common P-serotype worldwide, while P2A[6] is uncommon and seen primarily in asymptomatic neonatal infections [Gentsch et al., 1996].

Our study patients represent the age group at high risk for severe dehydrating rotavirus infection. Eight (47%) were less than 12 months of age and 8 (89%) of those tested showed no neutralizing antibody in their acute sera, suggesting that they might represent primary infections. However, this might not be true for all since most had low but detectable levels of anti-VP6 in

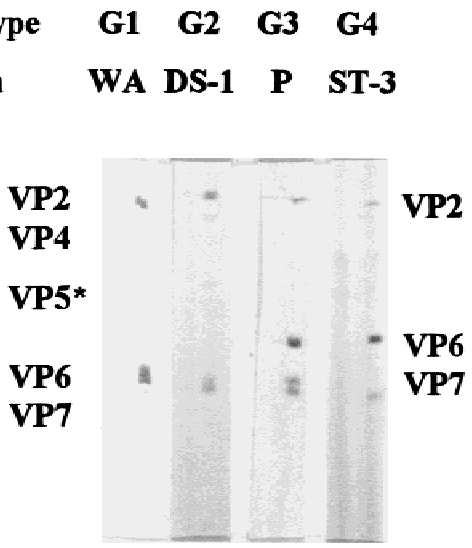

Fig. 2. Representative IgG responses following rotavirus serotype G4, P1A[8] infection. Note that anti-VP2 and anti-VP6 are present against all four serotypes. Anti-VP4 is present against serotype G2, P1B[4] (heterotypic), and anti-VP7 is present against serotype G4, P2A[6] (homotypic); G3, P1A[8]; and G1, P1A[8] (heterotypic). Note also that the relative migration of proteins is different for G1, G2 and G3, G4.

TABLE IV. IgG and IgA Immunoblot Responses to Viral Proteins of G1–G4 Serotype Viruses Following Rotavirus Serotype G4, P2A[6] Infection in Two Children

IgG (G-type/P-type) strain	Number responding to each protein[a]						
	VP1	VP2	VP4	VP5*	VP6	VP7	VP8*
WA (G1, P1A[8])	1	1	1	1	2	1	
DS-1 (G2, P1B[8])		1		1	1		
P (G3, P1A[8])		1		2	2	2	
ST-3 (G4, P2A[6])		1		1	1	**1**	

[a]Numbers in boldface = homologous response.

IgA (G-type/P-type) strain	Number responding to each protein[a]						
	VP1	VP2	VP4	VP5*	VP6	VP7	VP8*
WA (G1, P1A[8])					2		
DS-1 (G2, P1B[8])					1		
P (G3, P1A[8])					2		
ST-3 (G4, P2A[6])				1	1		

[a]Numbers in boldface = homologous response.

their acute sera. For the youngest patients (e.g., <6 months), this could represent maternally transferred antibodies, but for the others it likely represents prior rotavirus infection. This should be considered when interpreting immune responses, especially as it relates to heterologous responses, as discussed below.

IgG and IgA humoral antibodies developed following rotavirus infection. We found IgG testing more informative with the responses directed against a greater repertoire of viral proteins than IgA. Grimwood et al. [1988] reported that of 44 children infected with rotavirus 91% developed serum IgG response while 84% developed an IgA response; furthermore, the level of IgG was about 10-fold higher than IgA. We did not measure IgA antirotavirus antibodies directly in intestinal secretions, but we suspect that they would have occurred more often than humoral IgA. The same study described that duodenal and fecal IgA responses developed in 84% and 77% of the patients, respectively, and that serum IgA had a 62% sensitivity and 83% positive predictive accuracy of duodenal IgA response.

The inner capsid proteins VP6 and VP2 were the most antigenic, with serologic responses present in the majority of patients against both homologous and heterologous strains. Neither of these proteins are associated with neutralizing antibodies. However, at least for anti-VP6 antibodies, experimental data suggest that their presence might have a protective effect against rotavirus infection [Burns et al., 1996; Hermann et al., 1996]. Also, a number of other structural proteins showed different degrees of antigenicity. The role of these proteins in the immune response is uncertain, though. NSP4 has recently been described to act as an enterotoxin, probably mediating the diarrhea induced by the rotavirus [Ball et al., 1996]. If antibodies are developed against this protein, and what their role might be in protection from the disease is unknown at this point. Because of its molecular weight (29 kDa), NSP4 would be expected to migrate close to VP8* (weight: 28 kDa). As shown in Table III, homotypic anti-VP8* was commonly seen following serotype G1 infection but not G4 infection. Our technique does not allow us to differentiate between antibodies directed against VP8* and NSP4. Of note, however, is that anti-VP8* has been previously described to have neutralizing activity of uncertain clinical significance [Ruggeri and Greenberg, 1991].

Homotypic anti-VP7 developed in most patients following infection with either serotype G1 (80%) or G4 (86%). Similar findings have been reported by Richardson and Bishop [1990] and Richardson et al. [1993] when analyzing the immune response following natural infection. Homotypic anti-VP4 antibodies were found in 60% of children following P1A[8] infection; 7 (70%) of 10 G1-infected and 2 (40%) of 5 G4-infected. No homotypic anti-VP4 antibodies were found following G4, P2A[6] infection (0 of 2). The findings for anti-VP4 responses for our G1-infected patients are similar to those of Richardson and Bishop [1990], who described 67% of their G1-infected patients developing anti-VP4 IgG antibodies. The latter study differs from ours, however, in that the authors noted that the majority of their G4-infected patients also developed homotypic anti-VP4 [Richardson et al., 1993]. The reason for these different results might be due to differences in the P-serotype of the viruses infecting their patients and those used to assay immune response. In addition, we found that heterotypic anti-VP7 or anti-VP4 were overall infrequent, except for the appearance of antibodies against VP7 of the G3, P1A[8] strain (7 of 7) following G4, P1A[8] infection. Since VP4 and VP7 are well known to induce neutralizing antibodies [Hoshino et al., 1985, 1988; Svensson et al., 1987; Gorziglia et al.,

1990], the pattern of occurrence of anti-VP7 and anti-VP4 in our study suggests that homotypic protection could develop in most, if not all, cases of primary rotavirus infection, while heterotypic protection is partial, i.e., it develops neither in all cases nor against all serotypes.

In addition to what has been discussed, several possible limitations of our findings should be noted. The preparation conditions of the immunoblot assay can denature proteins, changing the conformation of epitopes and leading to underestimation of the presence of antibodies. Also, our assay may have underestimated the presence of serotype G2 anti-VP7, since the representative strain (DS-1) repeatedly showed only small amounts of the outer capsid protein VP7, even though VP4 was readily seen. We do not know if this fact is related to defective expression of VP7, alteration of VP7 by the preparation technique, or low antigenicity of the protein.

In conclusion, the serologic response following natural infection with rotavirus serotypes G1, P1A[8]; G4, P1A[8]; and G4, P2A[6] includes both the IgG and the IgA class of antibodies and is directed against core (VP2), inner capsid (VP6), and outer capsid (VP7 and VP4) proteins. Of the antibodies with neutralizing potential, anti-VP7 and anti-VP4 IgG are frequent against homologous strains. Heterotypic responses are partial. The role of these antibodies in clinical protection still needs to be elucidated in order to better define the necessary components for an effective rotavirus vaccine.

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